Research Note

Effect of Selection for Resistance and Susceptibility to Viral Diseases on Concentrations of Dopamine and Immunological Parameters in Six-Week-Old Chickens

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ABSTRACT White Leghorn chickens were inbred respectively from their parent lines, which were diversely selected for resistance (line 6_3) or susceptibility (lines 7_2 and $15I_5$) to Marek's disease and lymphoid leukosis. The differences in disease resistance may have been due to differential regulation of immune and neuroendocrine homeostasis. At 5 wk of age, chickens from the same line were randomly assigned to cages at 4 birds per cage. Blood samples were collected from the chickens at 6 wk of age (n = 10/line). Subsets of T lymphocytes ($CD4^+$ and $CD8^+$) and B cells were measured using flow cytometry. Concentrations of plasma IgG and dopamine were quantified with ELISA and HPLC assay, respectively. Line 6_3 chickens had a higher percentage of $CD8^+$ cells but not $CD4^+$ cells than the chickens of the lines 7_2 and $15I_5$ ($P < 0.01$). In contrast, both lines 7_2 and $15I_5$ had a greater percentage of B cells ($P < 0.01$). The concentrations of plasma IgG and dopamine were also regulated differently among the lines; both were in an order of $7_2 > 15I_5 > 6_3$ ($P < 0.05$ and $P < 0.01$, respectively). These results suggested that genetic selection for disease resistance also directly or indirectly modified the corresponding genetic components that govern the immune and neuroendocrine systems. The genetic lines of chickens may be used as animal models for investigation of the cellular mechanisms of genetic-environmental interactions on disease resistance.

Key words: chicken, genetic selection, dopamine, immunoglobulin G, viral disease

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INTRODUCTION

Genes determine functions of the neuroendocrine and immunological systems that affect an animal's ability to cope with stress, resistance to disease, and productivity. As a strategy to enhance resistance to diseases, chickens have been selected for the phenotypic characteristics associated with resistance (line 6_3) or susceptibility (lines 7_2 and $15I_5$) to Marek's disease and lymphoid leukosis (Bacon, 2002). The differences in disease resistance among the lines may be related to differences in expression of genes controlling the neuroendocrine system and immunity. Albers (1993) indicated that resistance to most diseases, including Marek's disease, has a low heritability, and defense against infectious diseases often requires resource allocation (Gross et al., 2002). In chickens as well as other animals, genetic basis of resistance to diseases is a multigenic trait regulated by the immune system and its interactions with many physiological and environmental factors (Zekarias et al., 2002; Fulton, 2004).

Early studies have shown that, compared with the chickens of 7_2 and $15I_5$, the line 6_3 chickens also have a lower frequency of aggressive behaviors, such as aggressive pecking and fights, in intraline (paired with the same line) and interline (paired with roosters from other lines) tests (Dennis et al., 2004). The findings further suggest that selective breeding in chickens based on 1 characteristic, such as disease resistance, could affect other genes or systems that associate with its line unique physiological characteristics in response to stimulations. The differences in resistance to viral diseases and social stress-induced aggressive behaviors among the lines may be reflected in differences in regulating the stress-buffering systems, such as the hypothalamus-pituitary-adrenal axis and neurotransmitter system (Cheng et al., 2001a,b), resulting in each line's unique characteristics of resistance to avian diseases (Bacon, 2002).

Abnormal blood and brain dopamine (**DA**) systems, such as changes of its concentrations, have been associated with the alterations of animals' ability to cope with stress (Snider and Kuchel, 1983; Van Loon, 1983), alterations of immunity (Basu and Dasgupta, 2000), and resistance to diseases (Li et al., 2005). Chickens of lines 7_2 and $15I_5$ appear to be lacking adequate strategies for coping with social stress and suffering from higher susceptibility to disease. This could be due to altered physiological

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homeostasis, including immunological and neuroendocrine systems, to compensate for the great costs of maintaining their social and health status, resulting from the artificial selection. However, this hypothesis has not been tested. The objective of the present study was to examine the effects of genetic selection for resistance to disease on plasma DA concentrations and immunological parameters.

MATERIALS AND METHODS

Genetic Lines

Three inbred lines, 6_3, 7_2, and $15I_5$, were examined in the current study. The original selection was started in 1939 from 10 distinct White Leghorn strains. In 1940, the basis for line 6_3 was developed by mating between males of strains 1, 2, 5, and 7 and females of strains 2 and 3; the basis for line 7_2 was from males of strains 1 and 7 and females of strains 2 and 7; and the basis for line $15I_5$ was from males of strains 2 and 7 and females of strains 2 and 3. For developing the current lines, breeders of each line were diversely selected from families that were most resistant or susceptible to viral diseases when exposed to an infected environment. In 1962, selection under a constant infection pressure was phased out, and an intense inbreeding program, a brother-sister mating program, was introduced; since then, breeds of the selected lines have been grown in isolated environments under a program for intense monitoring pathogens. The selective program resulted in 3 inbred chicken lines (i.e., line 6_3, which was resistant and lines 7_2 and $15I_5$, which were susceptible to Marek's disease and lymphoid leukosis (Waters, 1945; Stone, 1975; Bacon et al., 2000; Bacon, 2002).

At 5 wk of age, chicks from the same genetic line (n = 20) were randomly assigned to 4 birds per cage with 2,000 cm^2 of floor space. Each cage was provided with 2 nipple drinkers, and birds were fed ad libitum with a standard grower diet (14% protein, 3% fat, 2,810 kcal/kg, and premixed vitamin and mineral supplements). The light schedule was 11L:13D daily. To avoid caging effects (i.e., stress effects resulting from transportation and handling), the examinations began when the birds were at 6 wk of age. Chicken care guidelines were in strict accordance with the rules and regulations set by Federation of Animal Science Societies (Savoy, IL), and all procedures were approved by the Institutional Animal Care and Use Committee at Purdue University.

Blood Sampling

At 6 wk of age, 2 birds from each cage were randomly taken for blood sampling (n = 10 per line). A 20-mL blood sample was collected through heart puncture using a 20-gauge needle within 2 min of removing the bird from its cage. The heparinized blood samples were centrifuged at $700 \times g$ for 15 min at 4°C. Plasma was used for analysis of IgG (also called IgY in chickens), and cells were used for analysis of circulating lymphocyte populations.

Flow Cytometry Analysis for Immunocompetent Cells

Flow cytometry analysis of the percentage of lymphocyte populations, including $CD4^+$, $CD8^+$, and B cells, was performed using the methods as described by Li et al. (1999) and Kliger et al. (2000). After removing the plasma, buffy coat solution (Life Technologies Inc., Frederick, MD) was added immediately to each sample tube. The mixed samples were centrifuged at $700 \times g$ for 40 min at 4°C. Leukocytes contained in the buffy coat medium were separated from any remaining red blood cells using red blood cell lysing solution (Life Technologies Inc.) by centrifugation at $700 \times g$ for 20 min at 4°C. The leukocytes were washed 2 times in 1640 medium by centrifugation for 10 min at $500 \times g$. The cell pellet was resuspended and washed 2 times in fluorescence-activated cell sorter buffer (Hanks' balanced salt solution without phenol red containing 0.2% NaN_3 and 3% fetal calf serum; Life Technologies Inc.). The cell density was counted using a Coulter Z1 Cell Counter (Coulter Inc., Kennesaw, GA) and was resuspended in the fluorescence-activated cell sorter medium at 1×10^6 cells/mL. Two hundred microliters of the cell suspension of each sample was added into separate tubes for phenotype determination using direct fluorescein isothiocyanate- and phycoerythrin-conjugated antibodies for $CD4^+$ and $CD8^+$ immune cells (Southern Biotechnology Associates Inc., Birmingham, AL), respectively. Based on previous findings, Bu-1 antigen represents a unique surface marker for all B cells and their precursors (Houssaint et al., 1989; Tregaskes et al., 1996). Anti-Bu-1 antibody (Southern Biotechnology Associates Inc.) was used for B-cell analysis in the present study. Based on a preliminary study, the cells were incubated for 1 h at 4°C with antibody concentrations as follows: 50 μL of 1:50, 1:100, and 1:100 dilutions for $CD4^+$, $CD8^+$, and B cells, respectively. After washing and centrifuging, cells were fixed with 1.0% paraformaldehyde, the percentage of labeled cells was determined using a Coulter XL MCL flow cytometer (Beckman Coulter Inc., Fullerton, CA) with a 488-nm air-cooled argon laser for excitation, a 525 band pass for fluorescein isothiocyanate labels, and a 575-nm band pass for phycoerythrin detection. Cell only was included as negative control. Results were analyzed using the system II software (Beckman Coulter, Inc.) and reported as the percentage of total live cells.

ELISA to Determine Plasma Concentrations of IgG

Concentrations of blood IgG were measured in duplicate using a chicken IgG ELISA quantitative kit (Bethyl Laboratories Inc., Montgomery, TX) with a modification based on the manufacturer's recommendations and the methods used by Yonash et al. (2002). Based on preliminary tests, a plasma dilution of 1:20,000 was used in the current study, and the control IgG standard curve was from 0 to 500 ng. Briefly, diluted samples and standard plasma were added separately into 96-well ELISA plates coated with affinity-purified goat anti-chicken IgG. After incubation, goat anti-chicken IgG conjugated to peroxidase was added at a dilution of 1:1,200 into each well. Two wells containing no test serum were included on each plate as negative controls. Following incubation and washing, the substrate solution for peroxidase (1:1, tetramethylbenzidine peroxide substrate A:peroxidase solution B) was added then treated with stopping reagent (2 *M* H_2SO_4). The enzyme-substrate reaction was read on a microplate spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT) using KC4 software (Bio-Tek Instruments Inc.), and the concentrations of plasma IgG were determined when compared with the IgG standard curve on each plate and converted to milligrams per milliliter.

HPLC Assay

The ESA plasma catecholamine analysis kit (ESA Biosciences Inc., Chelmsford, MA) was used to measure blood concentrations of DA. Duplicate plasma samples were acidified and deproteinized with 4 *M* perchloric acid. After centrifugation, the acid supernatants and internal standard dihydroxybenzylamine were added and absorbed onto an alumina minicolumn to bind the DA. The columns were then rinsed and eluted with the solutions supplied by the company. Following injection of eluents into the reverse-phase columns, catechols were detected by liquid chromatography with electrochemical detection. The mobile phase (75 m*M* Na_2HPO_4, 1.7 m*M* 1-octanesulfonic acid, 25 μ*M* EDTA, 10% CH_3CN, and 100 μL/L triethylamine, adjusted to pH 3.00 with H_3PO_4) flow rate was 1.3 mL/min. Dopamine concentrations were calculated from a reference curve and were presented as nanograms per milliliter.

Statistical Analysis

The experimental design was completely randomized, with the treatment and lines as the main effects. All data were analyzed by 1-way ANOVA with SAS GLM procedure (SAS Institute, 1992), followed by paired *t*-tests. A level of $P < 0.05$ was accepted as statistically significant.

RESULTS AND DISCUSSION

Inbred chicken lines 6_3, 7_2, and $15I_5$ were from the parent lines, which were diversely selected for resistance or susceptibility to Marek's disease and lymphoid leukosis under a constant infection pressure (Bacon, 2002). Compared with chickens of lines $15I_5$ and 7_2, line 6_3 chickens had a higher percentage of $CD8^+$ cells but a lower percentage of B cells and lower levels of plasma IgG and DA concentrations. The available data further indicate that genetic selection on phenotypes of disease resistance might also have directly or indirectly altered the corresponding genetic components that govern chickens' physiological homeostasis.

The genetic basis of the differences in subpopulations of lymphocytes may be reflected in its unique line characteristics in resistance or susceptibility to Marek's disease and lymphoid leukosis. Compared with line 7_2 and $15I_5$ chickens, the line 6_3 chickens may have a more efficient cell-mediated immunity, as evidenced by a greater percentage of circulating $CD8^+$ cells (cytotoxic T lymphocytes or killer cells), which was much higher in the line 6_3 than those in both lines 7_2 and $15I_5$ ($P < 0.01$, Table 1). Previous studies have shown that $CD8^+$ cells are critical for protective immunity against various pathogens (Morrot and Zavala, 2004; Alexander-Miller, 2005), especially in eradicating infected cells and tumor cells via its cytotoxic functions (Levinson and Jawetz, 1996; Baz et al., 2005; Tang et al., 2005).

The great percentage of $CD8^+$ cells in the line 6_3 chickens could be due to their significantly high resistance to Marek's disease induced by Marek's disease virus (Witter et al., 2005). In support of this hypothesis, Omar and Schat (1997), Morimura et al. (1999), and Chang et al. (2002) found that $CD8^+$ T cells play important roles in removal of serotype 1 Marek's disease virus in chickens. Similar to the current findings, Burgess et al. (2001) reported that, following strain HPRS-16 Marek's disease virus challenge, line 6_3 had infiltrating $CD8^+$ cells with smaller size of lesions compared with both the line $15I_5$ and line 7_2. In their study, the rank of susceptibility determined by clinical signs and pathology were $7_2 > 15I_5 > 6_3 > N$. The N is another chicken line resistant to Marek's disease. In contrast, $CD8^+$ cells were downregulated in the Marek's-infected chickens (Morimura et al., 1995, 1996). These results are consistent with the current findings that the characteristics of lines 7_2 and $15I_5$ in susceptibility to Marek's disease may be related to their low percentages of circulating $CD8^+$ cells.

In contrast to line 6_3, both lines 7_2 and $15I_5$ had greater concentrations of IgG and a greater percentage of B cells, which were in order $7_2 \geq 15I_5 > 6_3$, respectively ($P < 0.01$, Tables 1 and 2). This data is consistent with the belief that alterations in IgG synthesis is a common response of B cells to genetic selection in animals, including both avian and mammalian species (Siegel et al., 1982; Van der Zijpp et al., 1983; Clark et al., 1996). The present study showed a negative correlation between total IgG concentrations and resistance to viral diseases, such as lymphoid leukosis and Marek's disease, in the present chicken lines. Higher concentrations of IgG were associated with a higher susceptibility to viral diseases of both lines 7_2 and $15I_5$. Similar to the present results, differences in physical and physiological characteristics have been found in other genetically selected strains; these characteristics include BW, feeding efficiency, and egg production in birds (Siegel et al., 1982; Van der Zijpp et al., 1983; Gross and Siegel, 1988); milk production in dairy cattle (Weigel et al., 1992), and levels of emotional stress in mice (Ozherelkov et al., 1985). In addition, chickens selected for high antibody (**HA**) and low antibody (**LA**) response to SRBC following immune challenge with Marek's disease virus showed that the HA line suffered higher mortality compared with the LA line (Tamaki, 1981; Okada and Yamamoto, 1987; Martin et al., 1989), and the HA

Table 1. Differences in subpopulations of lymphocytes in chickens differing in resistance to viral diseases

Line[1]	CD4$^+$ cells (% positive)	CD8$^+$ cells (% positive)	CD4$^+$:CD8	B cells (% positive)
6_3	43.3 ± 4.6	23.3^a ± 1.9	2.3^b	6.1^b ± 0.8
7_2	47.4 ± 2.6	9.1^b ± 1.2	6.0^a	16.9^a ± 1.9
$15I_5$	33.2 ± 3.8	10.0^b ± 2.1	5.2^a	14.1^a ± 1.3

[a,b]Means within a column with no common superscript differ significantly ($P < 0.01$; n = 10/line).

[1]The 6_3 as well as 7_2 and $15I_5$ line were inbred for resistance and susceptibility to avian disease, respectively.

line was more sensitive to *Escherichia coli*, *Pasteurella multocida*, or both than the LA line (Gross et al., 1980; Dunnington et al., 1991). The reason for the different regulation of IgG in the selected lines remains unclear, but it could be the same cellular mechanisms as that reported in the HA and LA line. The results indicated that selection for total antibody production in the HA and LA lines had altered the number of antibody-producing cells or different subset cells involved (Martin et al., 1989; Sarker et al., 1999). For example, the HA line, compared with the LA line, had a reduced effectiveness of macrophages (Gross and Siegel, 1988), lower proliferation of T cells (Shanks et al., 2000), and lesser immunological memory (Boa-Amponsem et al., 1999), which caused the changes that may have produced suitable conditions characterized by the production of nonantigen-specific antibodies (Van Eerden et al., 2004), low affinity and self-reactive antibodies (Doria et al., 1997), or both.

Dopamine plays an important role in regulating immunity by neuroimmune interactions (Qiu et al., 1996; Basu and Dasgupta, 2000). In agreement with the hypothesis, the current and previous data showed that 6_3 chickens bred for higher resistance to avian diseases had a lower concentration of plasma DA compared with the lines 7_2 and $15I_5$ selected for susceptibility to avian diseases ($P < 0.01$ and $P < 0.05$, respectively, Table 2). Similar to the current negative correlation between DA concentrations and susceptibility to disease, Li et al. (2005) reported that administration of DA suppresses the immune system and, in turn, promotes susceptibility to pathogens. In addition, Saha et al. (2001) found that stress-induced elevation of plasma DA significantly inhibited the proliferation and cytotoxicity of T cells (CD8$^+$ cells) in carcinoma patients, which is in agreement with the current findings [i.e., chickens of lines 7_2 and $15I_5$, selected for susceptibility to viral-induced tumors, had a low percentage of CD8$^+$ cells (Table 1)]. These results indicate that selectively breeding animals based on 1 characteristic, such as disease resistance, could affect other genes or systems that are associated with physiological characteristics. The selection may directly or indirectly affect the regulations of the dopaminergic system and, in turn, activation of the dopaminergic system to regulate immunity in favor of disease resistance in the line 63 chickens.

Table 2. Differences in blood concentrations of IgG and dopamine in chickens differing in resistance to viral disease

Line[1]	IgG (mg/mL)	Dopamine (ng/mL)
6_3	2.3^c ± 0.3	0.74^c ± 0.17
7_2	6.6^a ± 1.1	2.51^a ± 0.65
$15I_5$	4.0^b ± 0.7	1.28^b ± 0.24

[a–c]Means within a column with no common superscript differ significantly ($P < 0.05$; n = 10/line).

[1]The 6_3 as well as 7_2 and $15I_5$ line were inbred for resistance and susceptibility to avian disease, respectively.

In conclusion, the present study demonstrated that genetic selection for avian disease resistance in chickens affected the regulation of the neuroendocrine and immune systems in their inbred offspring. There were line differences for blood concentrations of DA, IgG, and percentage of CD4$^+$ cells, CD8$^+$ cells, and B cells between the disease resistant and susceptible lines. Those differences might be associated with the unique characteristics of lines in disease resistance. The unique homeostatic characteristics of each selected line may provide a useful animal model for investigation of the molecular and cellular mechanisms of disease resistance in poultry.

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